Relationships between otolith microstructure, microchemistry, and early life history events in Dover sole, *Microstomus pacificus*

Christopher L. Toole
Department of Fisheries and Wildlife
104 Nash Hall, Oregon State University
Corvallis, OR 97331-3803
Present address: Environmental and Technical Services Division
National Marine Fisheries Service
911 NE 11th Ave., Suite 620, Portland, OR 97232

Douglas F. Markle
Phillip M. Harris
Department of Fisheries and Wildlife
104 Nash Hall, Oregon State University
Corvallis, OR 97331-3803

Abstract.—Patterns of otolith microstructure and microchemistry (Sr/Ca ratios) are described in larval and juvenile Dover sole and related to developmental events and habitat. The initiation of metamorphosis is associated with a transition from clear (protein rich) to opaque (protein poor) material and formation of the first accessory primordium. Settlement is associated with the point at which growth from accessory primordia completely encloses growth from the central primordium and Sr/Ca ratio is minimal. No discrete otolith landmarks coincide with termination of metamorphosis.

Contrary to other flatfish that have a more abrupt metamorphosis, accessory primordia do not form until the left eye traverses the middorsal ridge of the cranium in Dover sole, an event that may occur months after the eye first reaches that position. A period of 70 days or more can separate the first- and last-formed accessory primordia in Dover sole, suggesting that all accessory primordia do not form in response to a single event.

A relationship between increments and days is validated for Stage 3–5 metamorphic and post-metamorphic stages. Duration of Stages 3 and 4 was in close agreement with predictions based on seasonal distributions. Duration of Stages 1 and 2, as determined by unvalidated increment counts, was about half as long as determined from seasonal distributions.

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Changes in otolith microstructure often correlate,and are presumed synchronous, with morphological and behavioral changes in larval and juvenile fish (Brothers and McFarland, 1981; Victor, 1982; Fowler, 1989; Ozawa and Penaflor, 1990; Gartner, 1991). The formation of secondary growth centers (accessory primordia) on flatfish otoliths is associated with metamorphosis in starry flounder, *Platichthys stellatus* (Campana, 1984a); plaice, *Pleuronectes platessa* (Alhossaini et al., 1989, Karakiri et al., 1989); California halibut, *Paralichthys californicus* (Kramer, 1991); and winter flounder, *Pseudopleuronectes americanus* (Sogard, 1991). Daily growth increments from accessory primordia are wider and are more likely composed of subdaily increments than those from the central primordium (Campana, 1984a; Karakiri et al., 1989; but also see Alhossaini et al., 1989).

Metamorphosis in flatfish includes a number of morphological and behavioral changes, most notably, eye migration and settlement from the water column to the sea floor. In most flatfish species, metamorphosis appears to be rapid; eye migration and settlement occur nearly simultaneously over a period of about 1 week (starry flounder, winter flounder, California halibut) to 3 weeks (plaice) (Ryland, 1966; Policansky, 1982; Campana, 1984a; Chambers and Leggett, 1987; Gadomski et al., 1992). Although timing of accessory primordium formation is not discussed in these studies, examination of published photographs suggests that the oldest and most recent accessory primordia are separated by only a few daily growth increments. In plaice, the earliest-formed accessory primordium (Alhossaini et al., 1989) or an unspecified accessory primordium (Karakiri et al., 1989) have been interpreted as settlement marks. In winter flounder, the earliest-formed accessory primordium has been interpreted as the point from which post-metamorphic age of an individual is determined (Sogard and Able, 1992). Counts and measurements of growth increments from accessory primordia to the otolith edge have been used to infer settle-

ment dates and post-settlement growth and mortality rates (Alhossaini et al., 1989; Karakiri et al., 1989).

In Dover sole (*Microstomus pacificus*), a commercially important northeast Pacific Ocean flatfish, metamorphosis differs from the rapid process described above. Markle et al. (1992) define five stages in Dover sole development. Stage 1 includes planktonic premetamorphic larvae from 6.1 to 58.5 mm standard length (SL). Eye migration begins when Stage-1 larvae are 10–15 mm SL but is arrested about midway through the process (Pearcy et al., 1977; Markle et al., 1992). Thus, Dover sole are optically asymmetrical during most of their planktonic life. Stage 2 includes planktonic metamorphic larvae from 42.3 to 60.4 mm SL, in which the eye has migrated past the mid-dorsal ridge and a pronounced shrinkage in body depth has begun. Stage 3 includes transitional metamorphic larvae from 40.7 to 74.9 mm SL, which are found in both the plankton and benthos. Stage-3 larvae are characterized by attainment of the adult configuration for a number of morphological characters. Stage 4 includes predominantly benthic, metamorphic larvae from 41.7 to 79.3 mm SL, which have formed a characteristic intestinal loop in the secondary body cavity. Stage 5 includes post-metamorphic juveniles from 48.9 mm SL to sexual maturity, which occurs at lengths greater than approximately 250 mm total length (Yoklavich and Pikitch, 1989; Hunter et al., 1992). Based on seasonal collections of staged larvae, Markle et al. (1992) estimated that duration of the metamorphic period (Stages 2, 3, and 4) is approximately 9 months.

The protracted metamorphic period in Dover sole provides an opportunity to examine changes in otolith structure and chemistry associated with each stage of metamorphosis. It may also be possible to distinguish between otolith landmarks associated with developmental processes and those associated with settlement from the water column to the bottom, because these events are not as coincidental in Dover sole as in other flatfish species. The objectives of this study were to 1) describe microstructure and microchemistry of Dover sole otoliths collected before, during, and after metamorphosis; 2) identify structural and chemical landmarks representing otolith growth during important morphological and behavioral transitions; 3) validate periodicity of increment formation in otoliths of metamorphic Stage-3 and Stage-4 larvae and post-metamorphic Stage-5 juveniles; and 4) using the otolith landmarks determined in objective 2 and increment counts between those landmarks and the otolith edge, re-examine the chronology of metamorphic events described in Markle et al. (1992).

Methods

Dover sole collections

Otoliths of six Stage-1, 103 Stage-3, 82 Stage-4, and 220 Stage-5 Dover sole were examined for microstructure and microchemical analysis. Specimens were obtained on various dates between 1987 and 1990 from bottom trawl collections off Oregon (Markle et al., 1992), opportunistic bottom trawl collections of commercial fishermen from Oregon and Washington, and midwater trawl collections off Oregon and central California[1] (Appendix 1).

The following measurements were taken to the nearest 0.1 mm on all specimens: standard length, body depth at anus (BD1A), and distance from snout to posterior extent of intestine (SINT). Weight of pat-dried specimens was determined to the nearest 0.1 g.

Otolith preparation and analysis for microstructure

Sagittae were removed after the fish were measured. Otoliths not immediately prepared for analysis were stored dry in vials. Terminology of otolith morphology follows Campana and Neilson (1985) and Secor et al. (1991), modified to account for features in Dover sole otoliths (Fig. 1 and Results section below). Left and right otoliths of Stage 3–5 Dover sole differ noticeably in shape (Fig. 1 and Results section below) and were analyzed separately.

The longest and shortest axes of sagittae from Stage-1 larvae were measured to the nearest 0.01 mm prior to mounting on slides in either a toluene-based medium (Histoclad) or an acrylic glue (Super Glue). Otoliths were then ground in the sagittal plane with 600-grit paper until growth increments became apparent.

Sagittae from Stage 3–5 Dover sole were cleaned in ethanol, air-dried, weighed to the nearest 0.01 mg, and measured along the anterior-posterior axis (maximum length) and along the dorsal-ventral axis (maximum width) to the nearest 0.01 mm (Table 1). The number of translucent rings (annuli) outside the clear central growth area (Fig. 1 and Results section below) was determined upon examination of whole otoliths under reflected light against a black background. Most otoliths were mounted on slides in a toluene-based medium (Histoclad) and the lateral face was ground in the

[1]Whipple, J. 1991. Progress in rockfish recruitment studies. U.S. Dep. Commer., NOAA, Natl. Mar. Fish. Serv., Southwest Fish. Sci. Center, P.O. Box 271, La Jolla, CA 92038. Admin. Rep. T-91-01, 57 p.

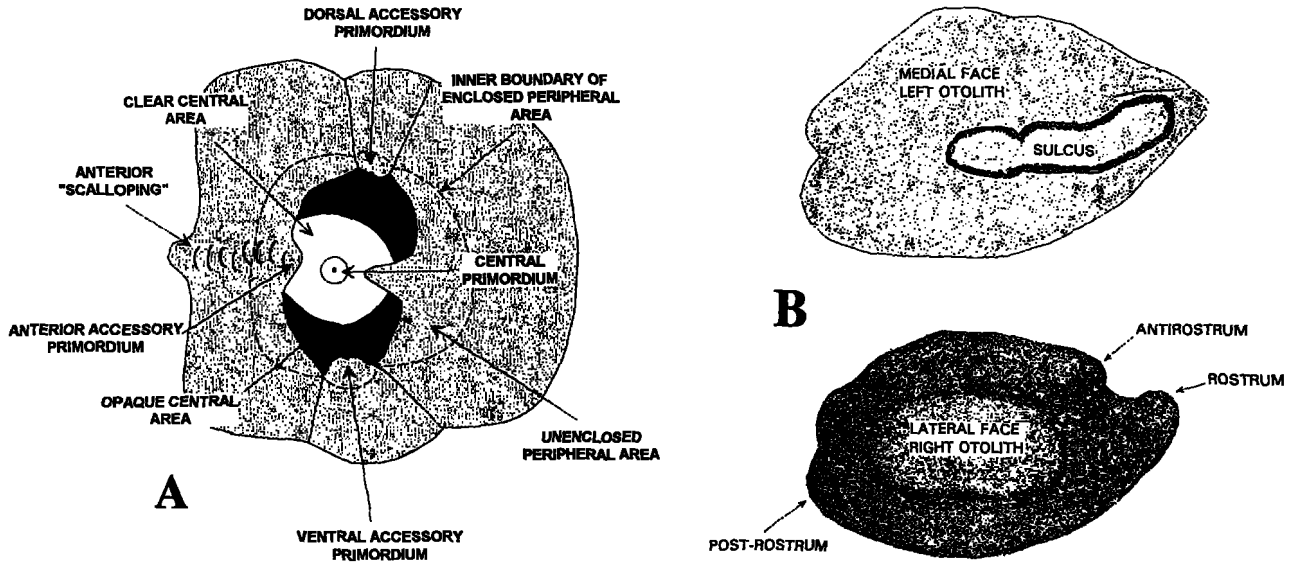

Figure 1

Schematic illustrations of Dover sole, *Microstomus pacificus*, otolith structure and orientation, showing terminology used in text. (**A**) Stage-4 left sagitta, sagittal section, lateral face towards the viewer, anterior to left. The anterior cavity is confluent with the anterior accessory primordium. (**B**) Orientation of left and right Stage-4 sagittae, anterior to right.

Table 1

Number of otoliths from Stage 3–5 benthic Dover sole, *Microstomus pacificus*, examined in study for each measurement or enumeration. AP = accessory primordium.

	Stage 3	Stage 4	Stage 5	Total
Right otoliths				
Whole otoliths				
Otolith length	81	53	215	349
Otolith width	81	53	215	349
Otolith weight	87	63	218	368
Number of annuli	87	63	209	359
Sagittal sections				
Diameter of clear central area	55	33	0	88
Number of AP	70	41	6	117
Increments since last AP (enclosed)	44	32	3	79
Increments since last AP (*not enc.*)	13	0	0	13
Increments since enclosure	51	46	3	100
Increments between checks	28	18	0	46
Left and right paired otolith comparisons				
Whole Otoliths				
Otolith length	67	50	198	315
Otolith width	67	50	196	313
Otolith weight	74	57	204	335
Sagittal sections				
Number of AP	41	22	0	63
Increments since last AP	23	12	0	35
First vs. last countable AP	14	11	0	25
Increments since enclosure	10	6	0	16
length anterior and posterior to AP	54	39	0	93

sagittal plane to the level of the central primordium. If resolution was not sufficient, the medial face was also ground to the central primordium. Otoliths from Dover sole >75 mm were ground and read progressively because all increments could not be observed in a single plane in sagittal section.

In order to examine otolith morphology in other planes, a subsample of otoliths from 17 Stage 3–5 Dover sole were embedded in low viscosity Spurr blocks (Haake et al., 1982) and ground to a level even with the central primordium in frontal or transverse sections. Ground sections were covered with a mounting medium and cover slip.

Additional otolith measurements were obtained from subsamples of Stage-3 and Stage-4 larvae (Table 1) to determine growth and development of features associated with metamorphic stages. The diameter of the clear central growth area (Fig. 1 and Results section below) of 88 right otoliths was measured to the nearest 0.02 mm on a black background at 50× with a dissecting scope and reflected light. Otoliths were then examined at 100, 400, and 1000× with a compound microscope and video monitor. At 100× we measured length of the otolith anterior and posterior to the central primordium to the nearest 0.01 mm in paired left and right otoliths from 93 larvae.

The number and position of accessory primordia, whether or not central primordium growth was completely enclosed by growth from accessory primordia, number of increments from accessory primordia to otolith edge, and number of increments from enclosure of central primordium growth to otolith edge were determined for all Stage-3 and Stage-4 larval otoliths in which the features were legible (Table 1). Similar information was obtained from 6 Stage-5 juveniles. Most counts and determinations were based on right otoliths; however, a subsample of left otoliths was also examined for paired comparisons (Table 1).

Counts of all growth increments from the first countable increment outside the central primordium to the otolith edge were made for five of six Stage-1 larval otoliths and for four Stage-3 larval otoliths exhibiting a variety of patterns of accessory primordium formation. Counts near the central primordium were made with 1000× light microscopy.

In all otoliths for which total increment counts were made and in approximately 20% of otoliths for which counts from accessory primordia were made, there were areas where accurate increment counts were not possible. In these areas, the number of increments was interpolated by linear approximation (Methot, 1983; Butler, 1989), based on the average widths of 5–20 increments on the distal or, preferably, both the proximal and distal sides of the uncountable area. Stage 3–5 larval otoliths with >5% interpolated increments were excluded from further analysis. We present the percentage of interpolated increments with total increment estimates for the few Stage-1 specimens examined.

Table 2
Rearing conditions of juvenile Dover sole, *Microstomus pacificus*, during the first validation experiment.

	Group 1	Group 2	Group 3
Collection depth	110 m	150 m and 163 m	108 m
Number injected	4	10	21
Number controls	13	0	0
Average weight[1]	5.4 g	3.4 g	4.5 g
Aquarium volume	152 L	76 L	114 L
Average density	0.036 g/L	0.045 g/L	0.039 g/L
Temperature	8°C	10°C	12°C

[1]Average for entire experiment, based on weights at death.

Validation of increment deposition rate

Two groups of juvenile Dover sole were given interperitoneal injections with a solution of 0.01 g oxytetracycline hydrochloride (OTC)/mL distilled water at a dose of 0.1 g OTC/kg fish weight (Campana and Neilson, 1982) to produce fluorescent marks on their otoliths. Dover sole in the first experimental group were held at three constant temperatures to evaluate temperature effects on increment formation. Those in the second experimental group were exposed to identical temperatures and were injected once in March and again in September to evaluate seasonal effects on increment formation.

First experiment An initial group of 48 juvenile Dover sole was collected at four stations off Cape Foulweather, Oregon, on 17 March 1990 (Table 2). Thirty-five fish were injected on that day and 13 served as uninjected controls to check for naturally occurring fluorescence. The fish were held in aquaria containing artificial seawater (Instant Ocean) and fed to satiation with *Tubifex* worms once per day in the morning. Fluorescent lights in the room were set to natural cycles, and aquaria were partially covered to reduce incoming light. Rearing conditions are summarized in Table 2.

Fish were sacrificed 21 (n=6), 26 (n=4) or 29 (n=9) days following initial injection. The remaining 29 fish were given a second injection of OTC 36 days after capture and were sacrificed 5 (n=6), 8 (n=10), or 12 (n=13) days after the second injection (i.e., 41, 44, or 48 days after initial injection).

Second experiment A second group of 10 juvenile Dover sole ranging from 55.3 to 118.3 mm was collected on 20 March 1991 at 77 m off Cape Foulweather, Oregon, and injected the following day. The fish were held in a flow-through filtered seawater tank. Ambient nearshore oceanic water entering the tank averaged 10.7°C (range: 8.7–14.6°C) during the experiment. Fish were exposed to diffused natural light and fed as in the first experiment. Seven days after injection, four fish, 55.3–63.9 mm, were sacrificed. The remaining six fish were individually marked with fin clips on 27 April. They were re-injected on 11 September, 174 days after first injection, and sacrificed 19 days later. Lengths ranged from 88.2 to 152.2 mm.

Otoliths from both groups of fish were prepared as described above and examined at 100–1000× on a Zeiss microscope equipped with a IV Fl epifluorescence condenser. The preparation was examined under full spectrum light to locate areas with distinct growth increments and the OTC band was then observed under ultraviolet light.

At least two documentary 35-mm slides of each otolith were taken: one showing the fluorescent band as well as the otolith edge and one showing increments under full-spectrum illumination. Each slide was projected and traced onto the same sheet of paper, resulting in a diagram that showed the location of the fluorescent band in relation to increments and other landmarks. The diagram served as documentation for increment counts as well as a guide for further examination with light microscopy. Photographs and counts were made at one to four locations on each otolith, usually at the rostrum, antirostrum, or post-rostrum (Fig. 1). Increment counts were generally made on right otoliths; left otoliths were used only if right otoliths were unavailable or illegible. All otoliths were read at least twice on separate dates by the same reader and the average was used in subsequent analyses. The range of estimates for each otolith and standard deviation of mean counts were also determined.

Results were analyzed by regressing number of increments between the proximal edge of the innermost fluorescent band and edge of the otolith against number of days since OTC injection. When a second fluorescent band was obvious and the area distal to the second band was illegible, counts were made between bands. To determine if all experimental groups could be combined into one regression, multiple regressions, including "dummy variables" corresponding to experimental groups, were analyzed with partial F-tests (Neter et al., 1989:364–370). The slope of the final regression model was compared to a slope of 1.0 (Neter et al., 1989).

Otolith preparation and analysis for microchemistry

Otoliths from two Stage-3 larvae (51.3 and 61.9 mm), three Stage-4 larvae (54.6, 57.1, and 65.7 mm), and two Stage-5 juvenile Dover sole (88.6 and 172.2 mm) were examined with a wavelength dispersive electron microprobe to determine if microchemical changes were associated with microstructural changes. Preparation and analytical techniques followed Toole and Nielsen (1992). Beam diameter was 5 μm, counting time for each element was 20 seconds, accelerating voltage was 15 kV, and current was 20 nA.

Results

Validation of increment deposition rate

First experiment Otoliths from uninjected control fish exhibited bright yellow-green fluorescence around the edge of the otolith and weaker fluorescence associated with strong checks and scratches. However, fluorescent marks observed on injected fish differed markedly in appearance. Twenty-seven injected fish had at least one otolith with one or two distinct fluorescent bands located inside the otolith edge (Fig. 2, A and B). Fluorescent bands extended over two to six increments.

Increments formed after capture were often less distinct than those formed in nature (Fig. 3), and four of 27 injected fish with fluorescent bands (14.8%) had otoliths with such poorly defined increments that they were considered unreadable and were eliminated from further analysis.

Both daily and subdaily increments (Campana and Neilson, 1985) were observed. For those otoliths in which both subdaily and larger increments could be counted in the same area, the ratio of small to large increments averaged 2.78 (n=10 fish, range 1.36–3.48). In most cases, larger growth increments could easily be distinguished from subdaily increments by adjusting the focus. However, in otoliths of two fish (7.4% of those with fluorescent bands), only subdaily increments could be observed. These fish were also eliminated from further analysis.

Second experiment Four Dover sole sacrificed 7 days after first injection had not fed. The remaining six fish began feeding approximately two weeks after the first OTC injection. Two of these fish grew rapidly throughout the experiment, whereas the other four fish ceased growing after about 20 weeks (Fig. 4).

Two of four Dover sole sacrificed 7 days after first injection and five of six fish sacrificed 19 days after second injection had at least one otolith with a

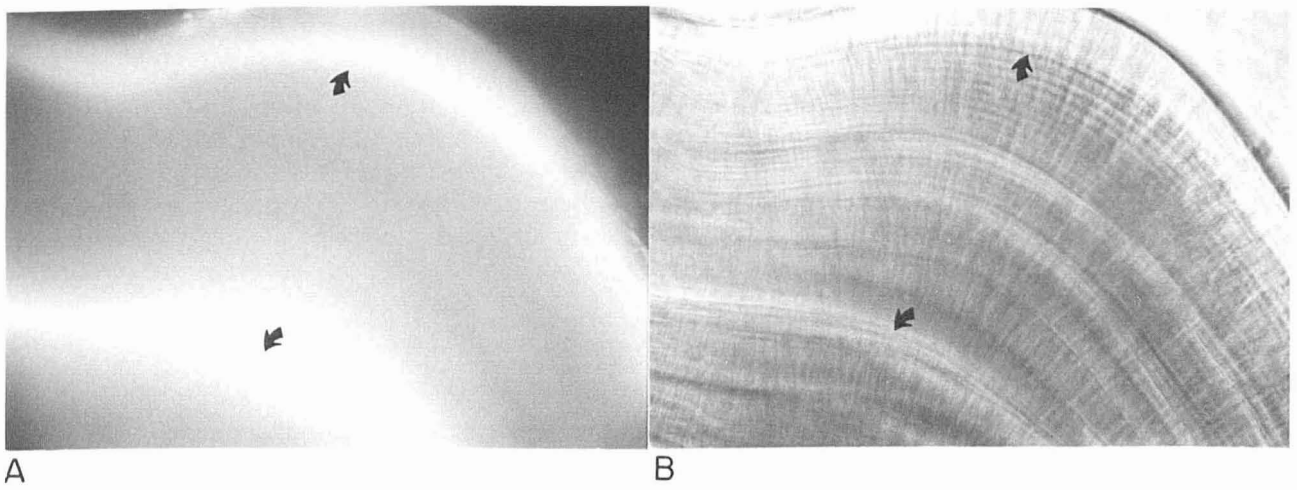

Figure 2

Sagittal section of right otolith from 67.2 mm Stage-4 Dover sole, *Microstomus pacificus*, larva marked with OTC and held in a 12°C aquarium for 48 days. Magnification = 400×. (**A**) Photograph taken with ultraviolet light; arrows show fluorescent OTC marks. (**B**) Same preparation taken with full-spectrum light; arrows show position of fluorescent OTC marks.

fluorescent mark and countable increments (70% of injected fish). Increment width was narrow, ranging from 0.63 to 1.88 µm. Because all counts underestimated the true number of days since injection and because larger marks were not apparent by adjusting focus, the marks were not equivalent to subdaily marks observed in the first experiment. The OTC mark deposited after first injection was not visible in fish held until second injection, so increments deposited during the entire 183-day period could not be counted.

Precision of counts Maximum and minimum increment counts for each otolith differed from mean counts by an average of 5.7% (Range=0–20%). Standard deviations associated with mean increment counts averaged 1.84 (Range=0–7.8).

Validation relationships Neither variances (Bartlett's test, B=1.48, P=0.11) nor mean observed/expected increment counts (ANOVA, df=27, F=2.21, P=0.10) differed between the five experimental groups of fish (three aquarium temperatures in the first experiment, March and September marking groups in the second experiment). Similarly, regressions of observed versus expected counts did not improve when a model containing separate slopes and intercepts for each experimental group was compared with a model containing separate intercepts and one slope (F=0.313, df=5,22,

Figure 3

Left otolith, anterior end of frontal section; from 68.0mm Stage-4 Dover sole, *Microstomus pacificus*, larva marked with OTC and held in a 10°C aquarium for 44 days. Arrow marks capture point, as identified by OTC fluorescence. Increments prior to capture are well-defined, whereas increments formed in aquarium are nearly indiscernible. Magnification = 400×.

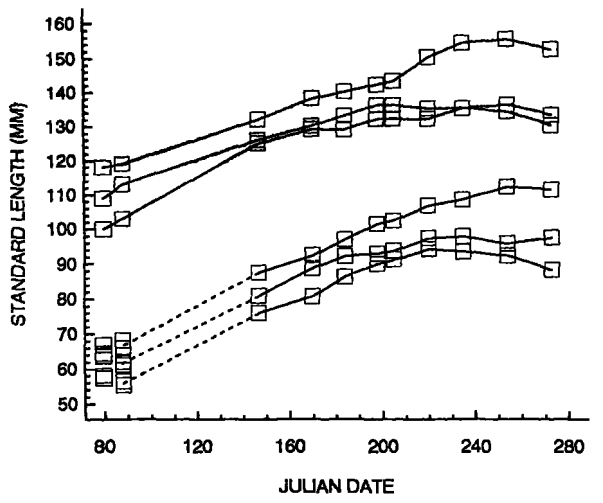

Figure 4

Relationship between standard length and calendar date of Dover sole, *Microstomus pacificus*, held in an ambient flow-through seawater tank, 21 March–30 September 1991 (Julian dates 80–272). Smaller fish were not marked initially; dotted lines indicate uncertainty regarding growth trajectories.

P=0.899) or when the model with separate intercepts and one slope was compared with a model containing one slope and one intercept (F=1.14, df=5,22, P=0.369), so all experimental groups were initially combined into one regression. However, inspection of residuals from that regression suggested that a greater error was associated with counts from Stage-5 juveniles than with counts from Stage-3 and Stage-4 larvae, regardless of experimental group. Mean ratios of observed:expected counts for fish <80 mm (0.924) than for fish ≥80 mm (0.718) (t-test, df=26, P=0.012). Therefore, each size group was treated separately in the final regression models.

The relationship for Dover sole <80 mm was

Days since marking = 3.81 + (0.962 * observed increments),

where n=21, r^2 = 0.85, $SE_{INTERCEPT}$ = 2.90, and SE_{SLOPE} = 0.091 (Fig. 5A). The calculated slope was not significantly different from 1.0 (t^*=0.419, df=26, P=0.34); however, the power of this test (Peterman, 1990) was too weak to conclude that increments were deposited daily (1–ß≈0.06; Rice, 1987, Neter et al., 1989).

The relationship for Dover sole ≥80 mm was

Days since marking = 6.32 + (1.026 * observed increments)

where n=7, r^2 = 0.93, $SE_{INTERCEPT}$ = 2.81, and SE_{SLOPE} = 0.122 (Fig. 5B). The calculated slope was not significantly different from 1.0 (t^*=0.211, df=6, P=0.42); however, the power of this test was also too weak to conclude that increments were deposited daily (1–ß≈0.05).

Error in the preceding regressions was due, in part, to poor resolution of laboratory-formed increments (Fig. 3; Campana and Neilson, 1985) and may therefore be greater than the error associated with ageing wild-captured fish. However, several resolution problems appeared common to otoliths of wild-captured and laboratory-reared fish: difficulty interpreting increments at the otolith margin, difficulty resolving increments around stress checks (Campana and Neilson, 1985) in certain regions of the otolith because of compression (and sometimes fusion) of increments, and occasional difficulty distinguishing daily and subdaily increments. For these reasons, use of both slope and intercept estimates was considered reasonable when back-calculating days from increments in otoliths of wild-captured fish. The same relationships were applied to increment counts outside the range of observations, which may introduce an unavoidable source of additional error.

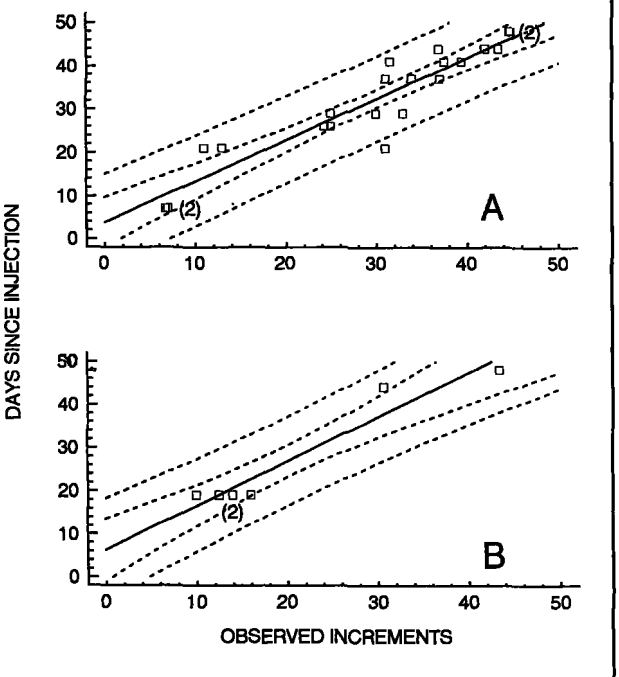

Figure 5

Relationship between days since OTC injection and number of growth increments in Dover sole, *Microstomus pacificus*, otoliths. Inner dashed lines are 95% confidence limits for mean response; outer dashed lines are 95% prediction limits. (**A**) Relationship for Stages 3 and 4 <80 mm SL. Formula: days since injection = 3.81 + (0.96 * observed increments); n=21; r^2=0.85. (**B**) Relationship for Stage-5 Dover sole ≥80 mm SL. Formula: days since injection = 6.32 + (1.03 * observed increments); n=7; r^2=0.93.

Table 3

Characteristics of five Stage-1 Dover sole, *Microstomus pacificus*, otoliths. Number of increments equals number observed plus number interpolated by linear approximation. The range of values presented for one larva reflects uncertainty in interpreting possible subdaily increments.

Standard length (mm)	Collection date	Maximum diameter (mm)	Number of increments	Percent interpolated (%)	Mean increment width (μm)
30.1	January, 1987	0.10	77	52	1.30
20.6	March, 1991	0.15	103	33	1.46
46.0	July, 1991	0.25	160–180	0	1.39–1.56
51.5	July, 1991	0.28	229	20	1.22
47.7	Oct., 1991	0.30	182	5	1.64

Structural patterns

Growth from central primordium Two prominent areas were seen within the field of growth emanating from the central primordium.

Clear central area Stage-1 larval otoliths were translucent and hemispheric (appearing nearly circular in sagittal section), with all growth emanating from the central primordium (Figs. 1 and 6). The left eye had previously migrated to the dorsal ridge of the cranium in all Stage-1 larvae examined. The diameter of Stage-1 larval otoliths ranged from 0.10 to 0.30 mm and the number of increments ranged from 77 to 229 (Table 3). When fully formed in Stage-3 larval otoliths, this clear central area was 0.24–0.49 mm (mean=0.39, n=60) and contained 187–230 increments (Table 4). Mean increment width in the clear central growth area ranged from 1.22 to 1.56 μm, and increment widths nearer the central primordium were approximately 0.5 μm, suggesting that, when counting increments, underestimation may have occurred owing to resolution limitations of light microscopy (Campana et al., 1987).

A structural feature of some Stage-1 larval otoliths was the presence of one or two conical cavities, which radiated towards the anterior and posterior edges (Fig. 7). Each cavity was enclosed laterally and medially and was open at the anterior or posterior end. The anterior cavity may be continuous with the sulcus, which was visible on one Stage-1 larval otolith.

Opaque central area An opaque area surrounded and was continuous with the clear central area in all Stage 3–5 larval otoliths (Figs. 1 and 8, A–D). Increments in this region were wider than increments in the clear central area (1.6–1.8 μm) and there was higher contrast between continuous and discontinuous zones within increments. Continuity of increments was interrupted by the conical cavities described previously and by accessory primordia. The number of increments in this area ranged from 46 to 95 (Table 4).

Growth from accessory primordia We recognized two zones of growth from accessory primordia, distinguished by the point at which growth from the central primor-

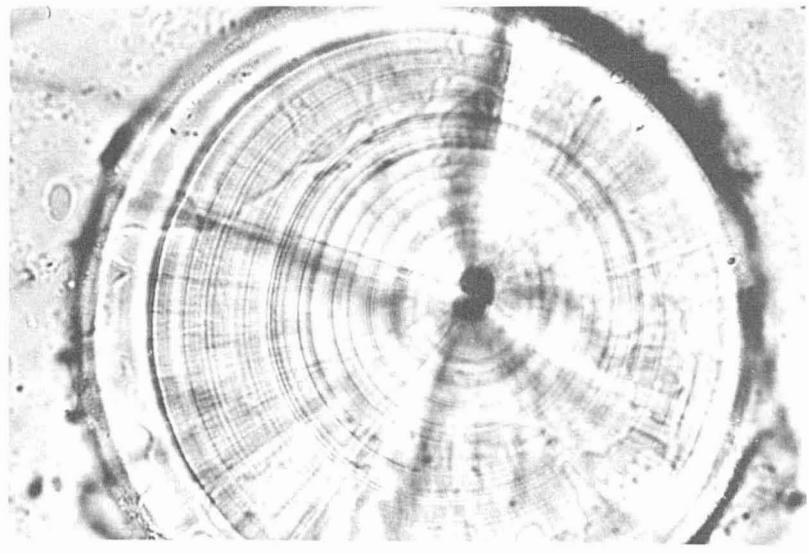

Figure 6

Sagitta from 20.6 mm Stage-1 Dover sole, *Microstomus pacificus*, larva collected on 1 April 1990 at NMFS Station 9003-35. Estimated number of increments was 103 (Table 3). Magnification = 1000×.

Table 4

Increments counted from central primordium to structural landmarks on four Stage-3 Dover sole, *Microstomus pacificus*, otoliths shown in Figure 8, A–D. Assigned dates (in parentheses) are determined from the increment:day regression for growth distal to accessory primordia. Periodicity of increments proximal to accessory primordia has not been validated. Ranges indicate uncertainty interpreting possible subdaily increments or determining exact location of the clear to opaque transition. The first distinct accessory primordium is not the first-formed accessory primordium. NP = not present; AP = accessory primordium.

	Standard length of larvae (mm)			
Landmark	55.6	46.9	64.9	55.5
Central primordium	0	0	0	0
Clear to opaque transition	187–213	205–219	≈230	≈200
First distinct AP	196–222 (19 Nov. 88)	NP	322 (3 Dec. 89)	206 (17 Dec. 89)
Last AP	224–250 (16 Dec. 88)	NP	338 (18 Dec. 89)	222 (1 Jan. 90)
Central primordium enclosure	NP	300–314 (20 Feb. 90)	376 (24 Jan. 90)	252 (30 Jan. 90)
Otolith edge	255–281	322–336	425	296
Capture date	19 Jan. 89	17 Mar. 90	17 Mar. 90	17 Mar. 90

dium was completely enclosed. This determination is based on sagittal sections at the level of the central primordium.

Unenclosed peripheral area

Otoliths of all Stage 3–5 larvae had at least two accessory primordia (AP), when viewed in sagittal section (Fig. 8, A–D). A maximum of seven AP were observed, although additional AP on the lateral surface of the otolith (Fig. 9) were obscured when otoliths were prepared in sagittal section. Accessory primordia always formed adjacent to growth from the central primordium, rather than adjacent to growth from previously formed AP. On right otoliths, AP were easier to discern than on left otoliths, possibly because the plane of growth changes at about the time of AP formation in left, but not right, otoliths (Fig. 10, A and B). Increments emanating from AP exhibited higher contrast and were wider (about 3.0 μm) than those emanating from the central primordium.

The most anterior AP appeared to form first and was closely associated with the transition from clear to opaque central areas. However, the exact origin of the anterior AP was usually impossible to discern because it merged

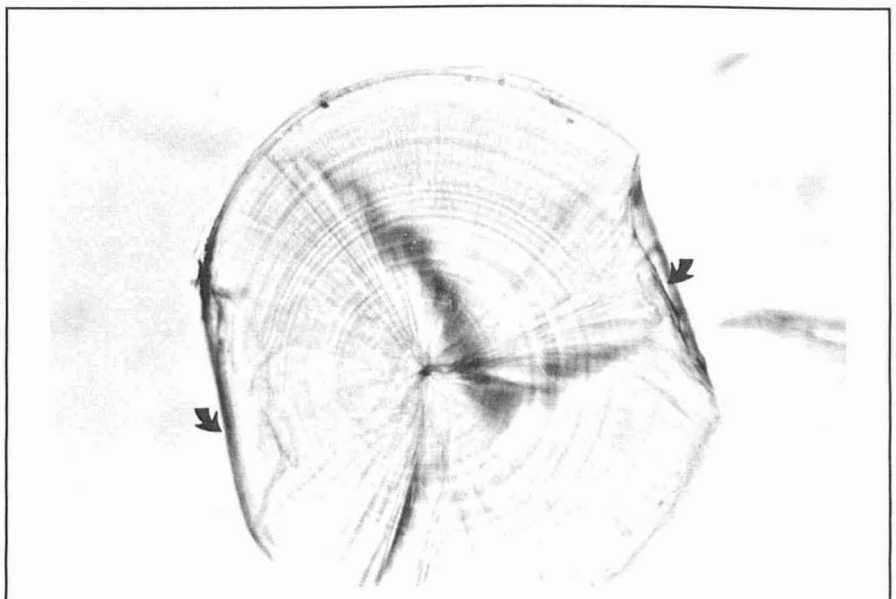

Figure 7

Sagitta from 51.5 mm Stage-1 Dover sole, *Microstomus pacificus*, larva collected on 12 July 1991 near Destruction Island, Washington. Anterior and posterior cavities are indicated by arrows. The lateral face of the otolith (surface toward viewer) has been ground to the level of the central primordium; the medial face has been left intact. Estimated number of increments was 229 (Table 3). Magnification = 400×.

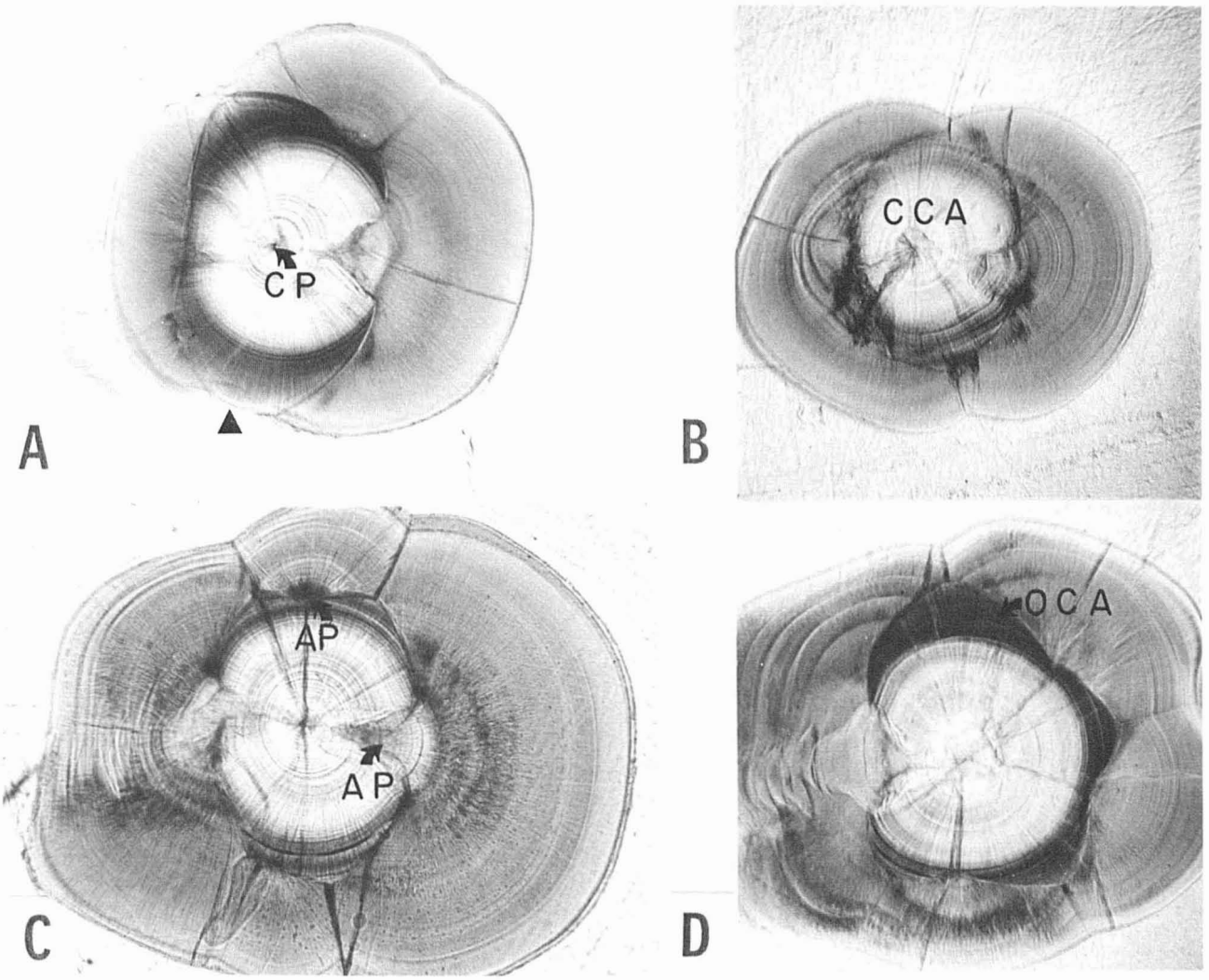

Figure 8

Stage-3 Dover sole, *Microstomus pacificus*, otoliths showing variations in accessory primordium formation; anterior to left, dorsal toward top in A–C, dorsal toward bottom in D. AP = accessory primordium, CCA = clear central area, CP = central primordium, OCA = opaque central area. (**A**) Left otolith of 55.6-mm larva collected 19 January 1989, showing unenclosed central primordium growth (indicated by triangle). Magnification = 100×. (**B**) Left otolith of 46.9-mm larva collected on 17 March 1990, showing two accessory primordia (anterior and posterior) completely enclosing growth from the central primordium. Magnification = 100×. (**C**) Left otolith of 64.9-mm larva collected 17 March 1990, showing growth from four accessory primordia (anterior, posterior, dorsal, and ventral) completely enclosing growth from the central primordium. Magnification = 100×. (**D**) Right otolith of 55.5-mm larva collected on 17 March 1990, showing growth from four accessory primordia (one anterior, two posterior, and one dorsal) completely enclosing growth from the central primordium. Magnification = 100×.

with the conical cavity described previously (Fig 8, A–D). This situation also applied to the origin of the most posterior AP in left otoliths (Fig. 8, A–C). The chronology of additional AP formation did not follow a consistent pattern. Dorsal and ventral AP were often associated with dark bands in the opaque central area (Fig.11).

Dorsal and ventral AP were formed 196–338 increments distal to the central primordium (Table 4). When increments were counted back from the otolith edge, dates of last AP formation varied between fish, ranging over a six-month period from October to March (Fig. 12). The number of days between the first-formed and last-formed dorsal and ventral AP averaged 28.8 (range 1–75, n=25). This range would be greater if it were possible to make accurate counts to the anterior AP.

Enclosed peripheral area Growth from accessory primordia completely enclosed growth from the cen-

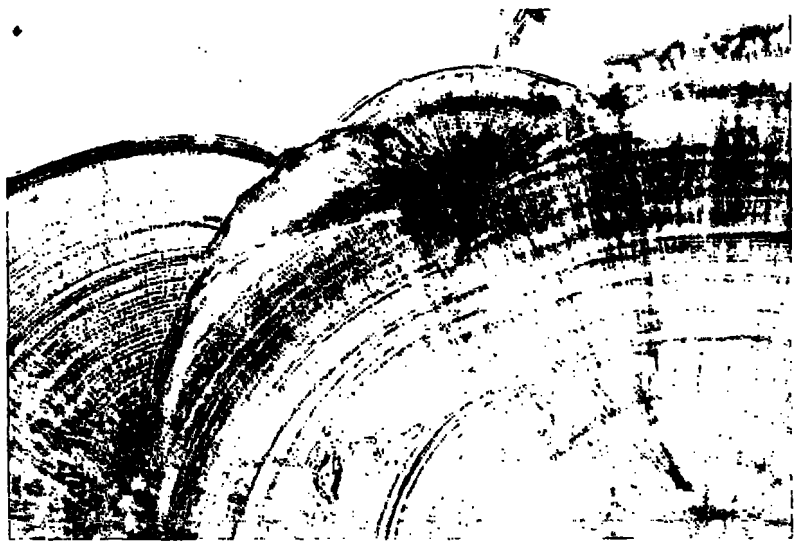

Figure 9
Transverse section of right otolith of 47.5-mm Stage-3 Dover sole, *Microstomus pacificus*, larva collected on 22 March 1989, showing recently-formed accessory primordium near medial surface (toward top) that would be undetectable in sagittal section. Magnification = 400×.

tral primordium in at least one otolith of 78% of benthic and 71.4% of pelagic Stage-3 larvae, 98.4% of benthic and 100% of pelagic Stage-4 larvae, and 100% of Stage-5 larvae, suggesting that most otoliths were enclosed before or during Stage 3. Enclosure of growth from the central primordium occurred an average of 33.7 days after formation of the last accessory primordium (range 0–87, n=108).

During Stages 3 and 4, SINT increases as the intestinal loop extends into the secondary body cavity and BD1A decreases as body depth shrinks (Markle et al., 1992). Consequently, fish with low SINT/BD1A ratios are presumably at an earlier stage in the metamorphic process than fish with higher ratios. Benthic Stage-3 larvae with at least one unenclosed otolith had a lower ratio of SINT/BD1A (mean=1.06) than benthic Stage-3 larvae with enclosed otoliths (mean=1.15) (t-test, df=46, P=0.0096), as did pelagic Stage-3 larvae (0.90 vs. 1.32, t-test, df=28, P=0.019), suggesting that Stage-3 larvae with unenclosed otoliths were at an earlier stage of development than Stage-3 larvae with enclosed otoliths.

Stage-3 larvae with enclosed otoliths were collected an average of 24.4 days after enclosure (range= 5.7–65.4, n=51) while Stage-4 larvae in the same collections averaged 51.9 days (range=9.6–125.9, n=46). The difference (27.5 days) may represent the average duration of Stage 3.

Otolith elongation along the anterior-posterior axis became more pronounced following enclosure, as did asymmetry between left and right otoliths. Left otoliths were heavier (paired t-test, df=335, P<0.001), longer (paired t-test, df=314, P<0.001) and wider (paired t-test, df=312, P<0.001) than right otoliths. In addition, the proportion of otolith length anterior to the central primordium was higher in right than left otoliths (t-test, df=92, P<0.001) by an average of 8.5% (Fig. 8, A–D). The number of accessory primordia tended to be higher on right than on left otoliths (paired t-test, df=62, P=0.036), while more increments formed after the last AP (paired t-test, df=34, P=0.006) and after the point of enclosure (paired t-test, df=15, P=0.011) on left than on right otoliths.

Stress checks became prominent in sagittal section following enclosure (Fig. 8, B–D). Enumeration of stress checks and determination of periodicity was difficult because checks were often discontinuous around the otolith and, even when continuous, the number of observable increments between checks varied regionally. However, mean periodicity of check formation in 46 Stage-3 and Stage-4 otoliths collected over a three-day period in March, 1990, suggested synchrony of check formation in otoliths of different fish and regularity in periodicity of check formation (Fig. 13). Calculated dates of check formation did not correspond to particular lunar phases. Number of days between checks ranged from 5.7 to 33.6, with a mode of 12.5 days and an average of 15.3 days (Fig. 14).

Other features of the enclosed peripheral zone in older fish were the development of a rostrum (Figs. 1 and 15) and formation of translucent growth zones (Fig. 16). All Stage-5 otoliths collected in January and March had at least one translucent growth zone. This first post-settlement translucent growth zone was initiated in late fall of the settlement year (240–293 days after formation of the last AP) and was completed the following spring (125 to 141 days later) in otoliths of three Stage-5 juveniles. Based on daily increment counts and seasonal deposition patterns, the first and subsequent translucent growth zones are interpreted as post-settlement annuli formed during slow winter growth periods (Fig. 17, page 746). This interpretation is consistent with Hagerman (1952) and Chilton and Beamish (1982).

Although there was little difference in weight between the largest Stage-4 and smallest Stage-5 Do-

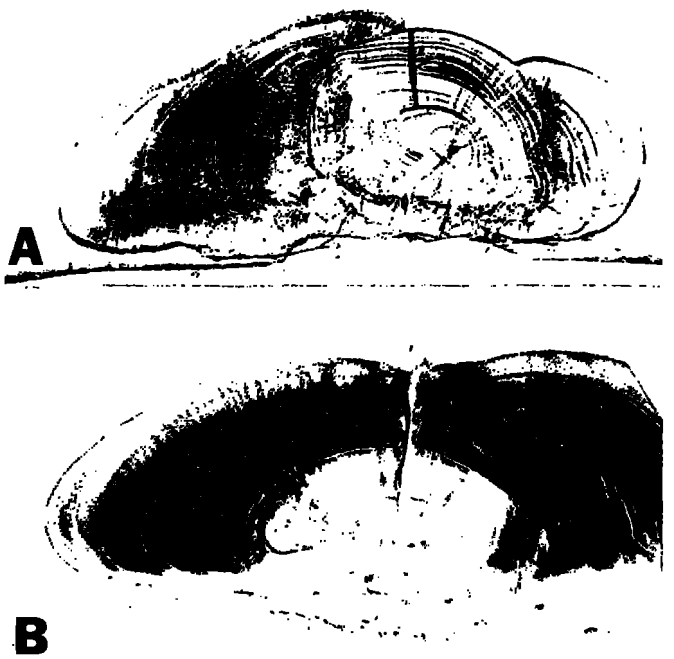

Figure 10
Frontal sections of left and right Dover sole, *Microstomus pacificus*, otoliths showing differences in orientation of the clear central growth area; anterior to right, lateral surface to top. (**A**) Left otolith from 58.0-mm Stage-3 larva collected on 16 March 1990. Central area tilts towards the lateral surface posteriorly and towards the medial surface anteriorly. (**B**) Right otolith from 64.9-mm Stage-3 larva collected on 17 March 1990. Central area is oriented in the same direction as surrounding peripheral growth.

ver sole collected in January and March, otolith weight in Stage-5 juveniles was nearly double that of Stage-4 larvae (Fig. 18, page 746). A discontinuity in the relationship between Stage 3–5 right otolith length and fish length during the first winter following settlement was also apparent (Fig. 19, page 746). This relationship was best described by a three-stanza segmented linear model (Bacon and Watts, 1971; Laidig et al., 1991). Parameter estimates in Table 5 (page 746) resulted in a good fit (r^2=0.955) and no discernable pattern in residuals. The first segment consisted of Stage-3 and Stage-4 larvae; the second, of Stage-4 larvae and Stage-5 juveniles; and the third, of only Stage-5 juveniles.

Microchemical patterns

Ratios of Sr/Ca exhibited similar patterns along transects through the otoliths of all larvae and juveniles examined (Figs. 20, A–B, page 747, and 21, page 748). An initial Sr/Ca spike (0.007–0.010) occurred approximately 48 μm from the central primordium, inside the clear central area. Following the spike, Sr/Ca levels within the clear central area fluctuated at intermediate levels (0.004–0.006). Sr/Ca ratios began a second, more gradual, decline beginning near the clear-to-opaque transition in the central area of the otolith, which also corresponded to the area in which the anterior AP was forming. This decline began in late summer or fall of the calendar year prior to settlement.

Sr/Ca ratios often reached minimum levels following formation of accessory primordia. In a 65.7-mm Stage-4 larval otolith (Fig. 20A), Sr/Ca ratios reached a minimum (0.002) at about the end of January. This minimum point was approximately 10 days beyond enclosure of growth from the central primordium and 31 days beyond the most recently formed accessory primordium. In a second Stage-4 larva, a minimum (0.003) was first reached in mid-De-

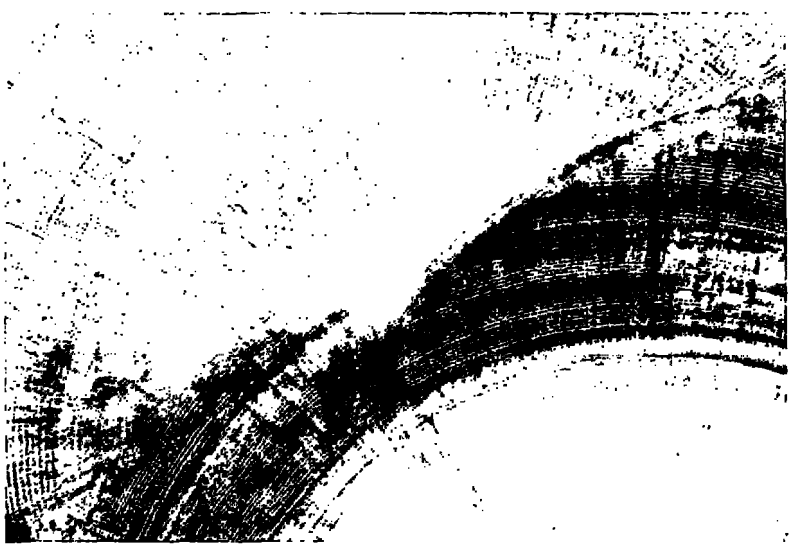

Figure 11
Posterior accessory primordium on right otolith of 58.0-mm Stage-3 Dover sole, *Microstomus pacificus*, larva collected on 16 March. Narrow dark band in the opaque central area is associated with origin of the accessory primordium. Magnification = 400×.

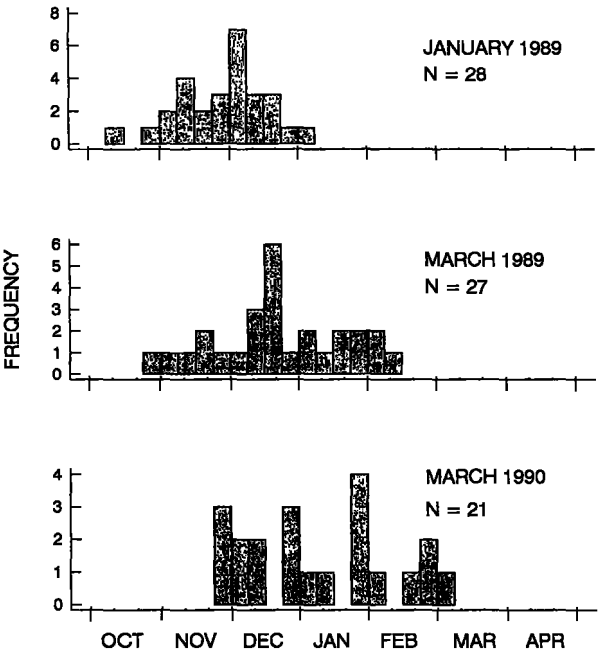

Figure 12

Dates of last accessory primordium formation on right otoliths of Stage-3 and Stage-4 Dover sole, *Microstomus pacificus*, larvae collected during three sampling cruises in 1989 and 1990. Only enclosed otoliths with three or more accessory primordia were considered because origins of single anterior and posterior accessory primordia are usually indistinct.

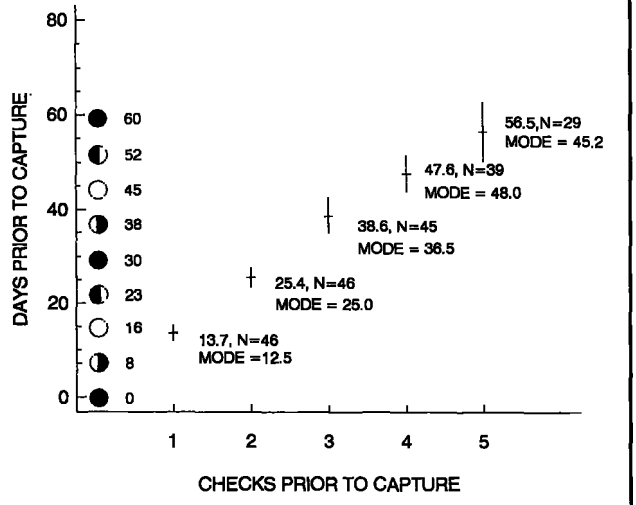

Figure 13

Relationship between the five most distal stress checks and number of days from their formation to the otolith edge (capture) in 46 Stage-3 and Stage-4 Dover sole, *Microstomus pacificus*, otoliths collected between 15–17 March 1990. Vertical bars represent 95% confidence intervals for means (horizontal bars). Additional error is associated with converting increments to days, with the regression described in the text. Corresponding lunar phases are displayed on the vertical axis.

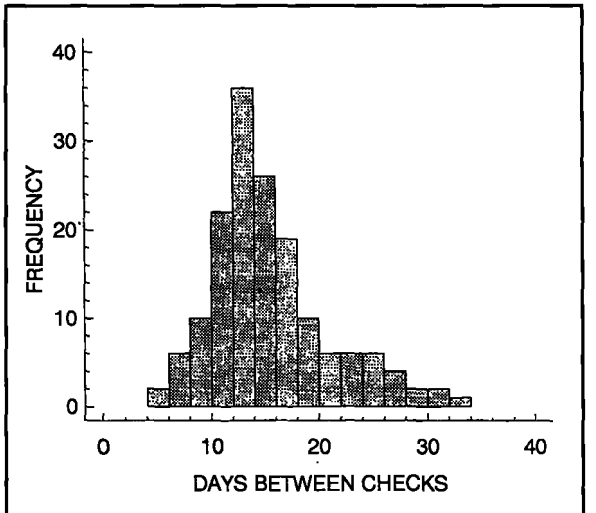

Figure 14

Frequency distribution of number of days between the five most distal stress checks on 46 Stage-3 and Stage-4 Dover sole, *Microstomus pacificus*, otoliths collected between 15–17 March 1990 (n=158).

cember. The ratio then rose and fell to a second minimum (0.003) in mid-January. In each of these larvae, the ratio rose again (0.003–0.0035) prior to capture in mid-March. Two Stage-3 larvae and a third Stage-4 larva did not exhibit distinct minima; rather, Sr/Ca ratios remained at about 0.0025 until capture. Sr/Ca ratios first reached this level prior to enclosure.

An 88.6-mm Stage-5 juvenile (Fig. 20B) with one peripheral annulus had narrow growth increments, making it difficult to determine exact ages associated with microprobe samples. However, the pattern proximal to AP was similar to that of larvae, with one 0.007 spike and a 0.004–0.006 peak near the center of the otolith, followed by a decline occurring prior to and during formation of accessory primordia. Sr/Ca ratios remained low for >200 days following formation of the last AP until a second 0.004–0.005 peak formed in the translucent annulus near the otolith edge, which represented capture in March.

A 172.2-mm juvenile, collected in November 1989 and judged to have three complete post-settlement annuli and a fourth one forming, had four Sr/Ca peaks, which corresponded to translucent annuli (Fig. 21), distal to accessory primordia.

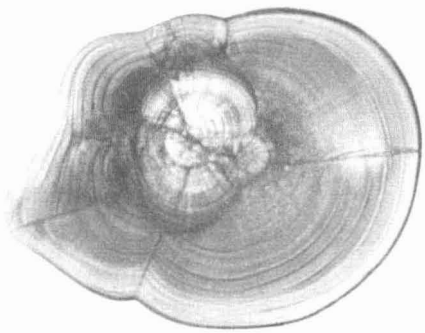

Figure 15
Left otolith of 65.0-mm Stage-4 Dover sole *Microstomus pacificus* larva collected on 17 March 1989; anterior to left. Note stress checks, which are clearest in the anterior field behind the anti-rostrum. Magnification = 50×.

Discussion

Relation of otolith microstructure to metamorphic stages

Premetamorphic larvae Sagittal otoliths of Stage-1 larvae between 20.6 and 51.5 mm were uniformly translucent and lacked accessory primordia. Since the left eye had migrated to the middorsal ridge of the cranium in each specimen, formation of AP is not triggered by the initiation of eye migration or movement to this position in Dover sole. Cavities noted in Stage-1 larval sagittae have not been described previously and their derivation and function are unknown.

Metamorphic larvae Because Stage-2 larval otoliths were not available, the transition from clear to opaque central areas and development of the first two accessory primordia cannot be attributed to this stage with certainty. However, because neither of these features were developed in Stage-1 larval otoliths, yet both were present in all Stage-3 larval otoliths, it is probable that they formed during Stage 2. Thus, the transition from clear to opaque growth and initiation of AP formation occur after the left eye moves beyond the dorsal ridge and metamorphosis (as defined in Markle et al., 1992) begins, but before larvae are first collected on the bottom.

Formation of the first accessory primordium in plaice and winter flounder also does not occur until the left eye has migrated at least to the dorsal ridge of the cranium (Alhossaini et al., 1989; Sogard, 1991). However, unlike Dover sole, AP in otoliths of these species can form before the eye has moved past that point. Comparisons with other flatfish species are more difficult. Accessory primordia form "at or shortly after metamorphosis" in starry flounder (Campana, 1984a) and "after metamorphosis" in California halibut (Kramer, 1991); however, the definition of metamorphosis relative

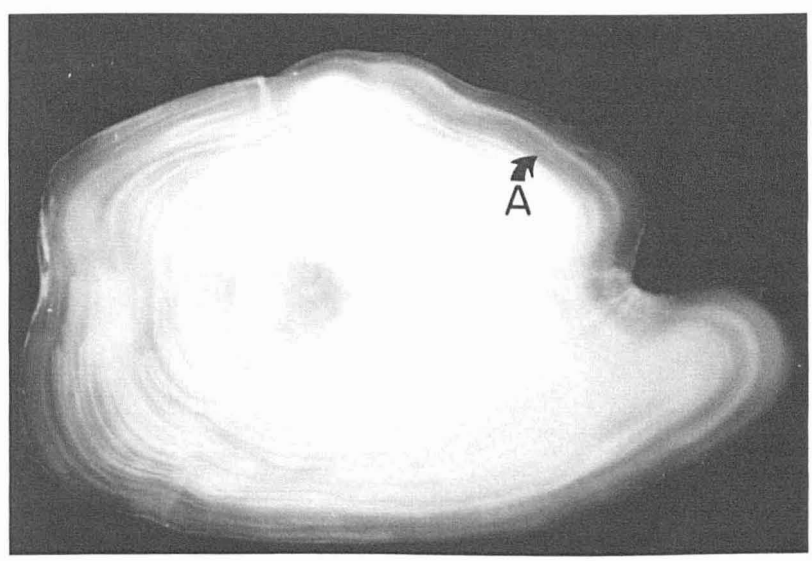

Figure 16
Right otolith of 100.4-mm Stage-5 juvenile Dover sole, *Microstomus pacificus*, caught on 18 March 1991 (anterior to right), photographed with reflected light against black background. Note the first annulus, which appears darker than the inner area. Magnification = 50×. A = annulus.

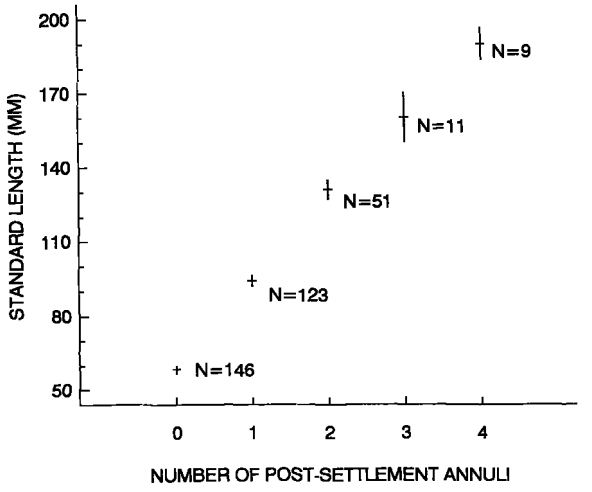

Figure 17

Relationship between standard length and number of post-settlement annuli in Dover sole, *Microstomus pacificus*, collected in January 1989, March 1989, and March 1990. Vertical bars represent 95% confidence intervals for means (horizontal bars).

to cranial morphology was not specified in these studies.

Formation of accessory primordia has also been noted in several non-pleuronectiform taxa (Brothers, 1984). Gartner (1991) described a clear to opaque transition with accompanying formation of AP in otoliths of myctophid species and suggested these features were

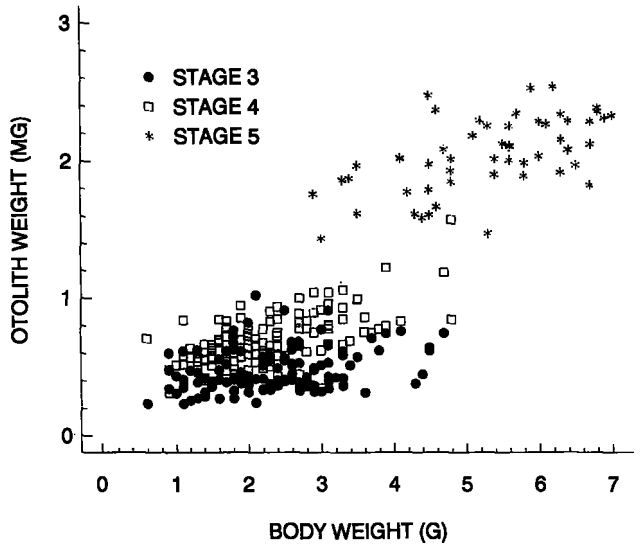

Figure 18

Relationship between weight of right otolith and body weight in Stages 3–5 Dover sole, *Microstomus pacificus*. Note change in the relationship during the transition from Stage 4 to Stage 5 (n=368).

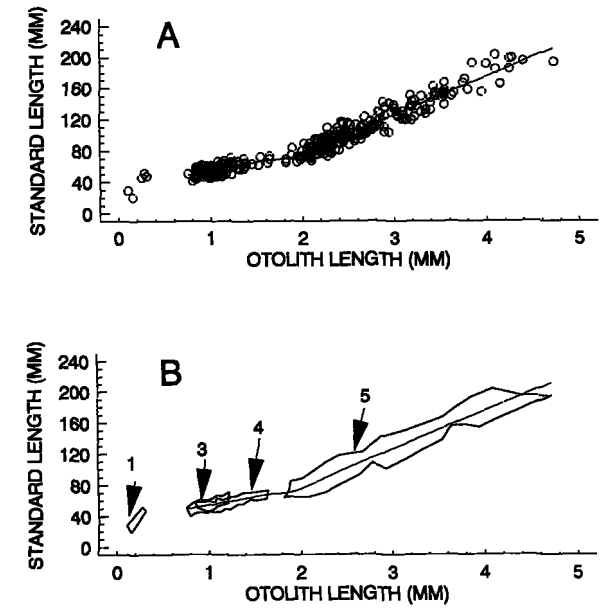

Figure 19

(**A**) Relationship between standard length and right otolith length in Stages 3–5 Dover sole, *Microstomus pacificus*. Formula and parameter estimates are included in Table 5. n = 351; r^2 = 0.955. Points representing standard length and right otolith diameter of five Stage-1 larvae are also included. While Stage-2 otoliths were not available, it appears that otolith length increased between Stage 1 and Stage 3, whereas fish length did not. (**B**) Polygons circumscribing areas bounded by fish in Stages 1 and 3–5.

Table 5

Estimated parameters for segmented linear regression model of the form

$$\text{Standard Length (mm)} = e + d_1(x-c_1) + d_2(x-c_1)s_1 + d_3(x-c_2)s_2,$$

where x = right otolith length (mm), s_i = [2*(1/(1+exp(–[x–c_i]/0.1)))]–1 (i.e., the logistic cumulative distribution function, used as a transition function between segments, with the constant 0.1 specifying a rapid transition between segments), c_i = fitted inflection points, and e and d_i = fitted parameters (n = 351, r^2 = 0.955).

Parameter	Estimate	Standard error
e	63.753	36.999
c_1	1.640	1.045
c_2	1.891	0.230
d_1	35.631	1.920
d_2	–6.485	40.950
d_3	19.999	41.233

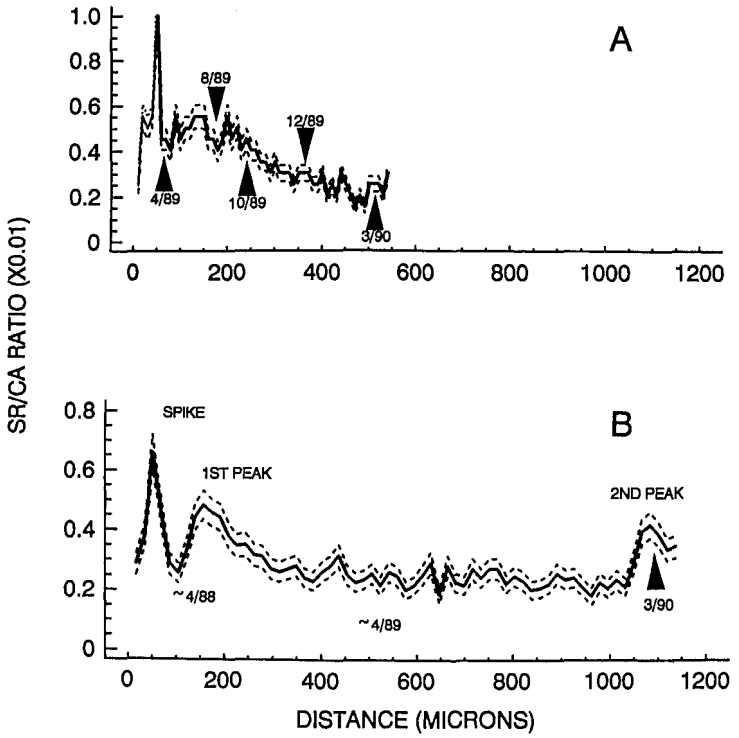

Figure 20

Relationship between Sr/Ca ratios and distance from central primordium towards edge in Dover sole, *Microstomus pacificus*, otoliths. Distances are not comparable between otoliths because transects were not oriented identically. Approximate dates are indicated, based on counts of increments between microprobe marks and the otolith edge. Dashed lines represent 2-σ measurement error, calculated as in Toole and Nielsen (1992). (**A**) 65.7-mm Stage-4 larva collected on 17 March 1990. (**B**) 88.6-mm Stage-5 juvenile collected on 17 March 1990. This fish had one annulus forming at the otolith edge, corresponding to the second Sr/Ca peak.

associated with a habitat transition from warm surface waters to colder midwater depths during the larval to juvenile transition. Dover sole could experience a similar temperature change at the time of AP formation, as discussed below.

Because growth from the central primordium was enclosed by growth from AP in otoliths of most Stage-3 larvae and because new AP were never observed distal to the point of enclosure, the last AP generally formed during either Stage 2 or Stage 3. The presence of very recently formed AP (<7 days) in some Stage-3 larvae and the possibility that additional AP could form in 22% (benthic) to 29% (pelagic) of unenclosed Stage-3 larval otoliths suggest that formation of the last accessory primordium may have occurred during Stage 3, rather than during Stage 2, in some individuals. Because Stage-3 larvae with unenclosed otoliths had lower SINT/BD1A ratios than Stage-3 larvae with enclosed otoliths, it appears that when the last AP does not form during Stage 2, it forms early in Stage 3. Thus, completion of AP formation may occur during the final stages of eye migration, when Dover sole larvae are still pelagic, or after completion of eye migration, shortly after Dover sole first settle to the bottom. In virtually all cases, it occurs before the intestinal loop begins to extend into the secondary body cavity.

Occasional formation of the last AP after settlement in Dover sole corresponds to observations of AP formation in other species. Alhossaini et al. (1989) described unenclosed otoliths of benthic plaice larvae, suggesting that AP formation may not be complete at settlement in that species. Karakiri et al. (1989) noted that the formation of accessory primordia "accompanies" the transition to a bottom-dwelling mode in plaice. If the formation of AP in California halibut "after metamorphosis" (Kramer, 1991) means "after eye migration is complete," then AP formation likely continues after settlement, because settling behavior of California halibut begins prior to initiation of eye migration (Gadomski et al., 1992).

Increased prominence of stress checks following enclosure may have been caused by a change in the otolith growth plane relative to the sectioning plane or by increased intensity of environmental cues that may induce check formation. Campana (1984b) correlated 15-day cycles of increment width and contrast in otoliths of intertidal starry flounder with similar cycles of tidally-induced temperature variation. Rosenberg (1982) described 14-day cycles of check formation in English sole (*Parophrys vetulus*), which first formed "at the beginning of the metamorphic period." An examination of Figure 2 of Rosenberg (1982) suggests that these checks were most obvious proximal to accessory primordia, as in Dover sole. The mean 15.3-day cycle of check formation observed in Dover sole may also be related to tidally induced temperature variation and larvae may be more strongly affected by these patterns following settlement, resulting in more prominent checks distal to the point of enclosure. However, the complex nature of rotational tidal currents in the vicinity of Dover sole nursery grounds precludes an evaluation of this hypothesis at present.

After accessory primordium formation, enclosure of growth from the central primordium, and formation of prominent checks, subsequent changes in larval otolith morphology were continuous, rather than discrete.

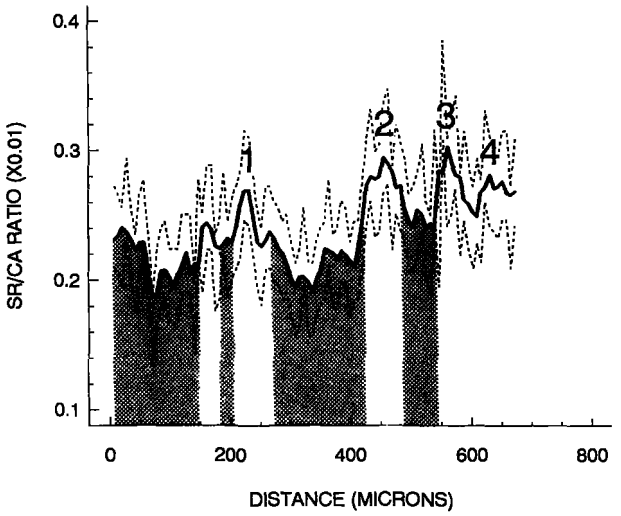

Figure 21

Relationship between Sr/Ca ratios and distance from boundary of enclosure towards edge in a 172.2-mm juvenile Dover sole, *Microstomus pacificus*, collected on 11 September 1989. The specimen had four post-settlement annuli and was prepared in transverse section. Solid line represents five-term running averages of Sr/Ca; dashed lines represent 2-σ measurement error, calculated as in Toole and Nielsen (1992); shaded areas represent opaque zones and unshaded areas represent translucent zones in the otoliths; and numbers above peaks represent post-settlement annuli.

There were no dramatic changes in otolith structure to mark the transition from Stage 3 to Stage 4.

Postmetamorphic juveniles Formation of the first post-settlement annulus occurred during the fall, several months after the transition from larval Stage 4 to juvenile Stage 5 that defines the end of metamorphosis (Markle et al., 1992). While the first and subsequent post-settlement annuli are useful for establishing the number of years since an individual settled and completed metamorphosis, they are not useful for determining either duration of the metamorphic period or the date associated with the end of metamorphosis. No other significant structural landmarks were associated with this stage.

Relation of otolith microchemistry to metamorphic stages

Premetamorphic larvae Within the clear central area formed during Stage 1, Dover sole otoliths exhibited high Sr/Ca levels relative to levels in adjacent opaque areas. Translucent zones of otoliths are rich in aragonite and have a poorly developed protein matrix, while opaque areas have higher protein concentrations (Dannevig, 1955; Williams and Bedford, 1974; Morales-Nin, 1987). Because organics are oxidized during beam exposure and 1:1 concentrations of CO_2 relative to cations are assumed in microprobe calculations (Toole and Nielsen, 1992), differences in concentration of organic materials could differentially affect accuracy of Ca and Sr measurements in translucent and opaque areas. However, Sr and Ca do not fractionate from one another in response to beam damage, so Sr/Ca ratios should not be affected by concentration of organics (Toole and Nielsen, 1992). Protein-poor areas of otoliths are generally associated with rapid growth (Dannevig, 1955; Williams and Bedford, 1974; Morales-Nin, 1987). However, many northeastern Pacific fishes, including adult Dover sole, form translucent zones (annuli) on otoliths during slow winter growth periods (Chilton and Beamish, 1982).

Sr/Ca ratios reflect the substitution of Sr for Ca in otolith aragonite, possibly owing to reduced ability to physiologically discriminate between Sr and Ca during biological precipitation (Radtke et al., 1990). The effect appears to be mediated through Sr concentration in the saccular endolymph, which in turn may be related to plasma protein levels (Kalish, 1989). Sr/Ca ratios have been described as inversely proportional to environmental temperature (Radtke, 1984, 1989; Townsend et al., 1989; Radtke et al., 1990) and growth rate (Sadovy and Severin, 1992), and directly proportional to age (Kalish, 1989), "stress" (Townsend et al., 1989), Sr concentration in ambient water (Kalish, 1990), and salinity of ambient water (Radtke et al., 1988). The latter two effects have only been documented under extreme conditions of seawater vs. freshwater residence. The effects of age described by Kalish (1989) were linked primarily to adults and may not apply to larvae and juveniles in this study.

Elevated Sr/Ca ratios in premetamorphic Dover sole otoliths could be related to a combination of low temperature, slow growth rate, and high "stress" (all of which may be expected to covary) if correlations observed in other studies apply to this species. However, confirmation of these effects in Stage-1 Dover sole is impractical, given available information. Temperatures experienced by premetamorphic larvae depend upon their distance offshore and depth, both of which are variable during this stage (Pearcy et al., 1977; Markle et al., 1992). Because periodicity of increment formation prior to AP formation has not been validated, growth rate cannot be determined with certainty. While it is possible that "stress" may be associated with developmental changes, the strong Sr/Ca spike approximately 48 µm from the central primordium appeared to occur subsequent to events such as hatching (at a length of ≈6 mm; Ahlstrom and Moser, 1975) and initiation of eye migration, caudal fin flexion, and growth

in body depth (at lengths of ≈10–15 mm; Pearcy et al., 1977; Markle et al., 1992), based on radii of otoliths from larvae of known lengths. (The radius of a 17-mm Dover sole larval otolith[2] was 38 μm and otolith radii of 20.6 and 30.1-mm larvae collected in the present study were 75 and 50 μm, respectively [Table 3].) Elevated Sr/Ca levels in the outer portion of the clear central area appeared to form near the termination of Stage 1, when larval morphology does not change (Markle et al., 1992).

Metamorphic larvae during Stage 2 and early Stage 3 Sr/Ca ratios declined in opaque otolith material presumably deposited during Stage 2 and early Stage 3. The decline either levelled prior to formation of the last AP or reached a minimum after enclosure of growth from the central primordium (Fig. 20A). Dover sole probably experienced progressively colder temperatures during this period as they moved from offshore waters onto the continental shelf and from the water column to the bottom (Pearcy et al., 1977; Landry et al., 1989; Markle et al., 1992). However, some larvae continue to move between the bottom and the water column, perhaps on a diel basis, throughout the metamorphic period (Markle et al., 1992), which would add further complexity to this pattern. Larvae appeared to lose mass and either did not grow in length or grew very little during this period (Markle et al., 1992). There was, however, a rapid increase in growth of otoliths, as evidenced by formation of accessory primordia with wide growth increments.

As with Sr/Ca ratios in otoliths of Stage-1 larvae, underlying causes of the observed pattern are unclear. If Sr/Ca ratios in Dover sole responded as in other species, decreasing temperature and slow somatic growth rate would be associated with increasing, rather than decreasing, Sr/Ca ratios. Otolith growth rate may be a better predictor of Sr/Ca ratio than somatic growth rate, and this may become apparent only when the two are uncoupled, as appears to occur during Stage 2 (Fig. 19).

Metamorphic larvae during Stages 3 and 4 Sr/Ca ratios remained low from formation of the last accessory primordium to formation of the first post-settlement annulus (Fig. 20B). Opaque otolith material formed during Stages 3 and 4, when most Dover sole were primarily, if not exclusively, on the bottom (Markle et al., 1992). Dover sole larvae increased in length and weight (Toole and Markle, unpubl. observations) and otolith length increased (Fig. 19) during this time, while bottom temperature decreased and nutrient levels increased following the onset of upwelling (Huyer et al., 1979; Landry et al., 1989). The correlation of low Sr/Ca ratios with rapid growth corresponds to observations of Sadovy and Severin (1992); the correlation of low Sr/Ca ratios with low temperature is contrary to observations of Radtke (1984, 1989), Townsend et al. (1989), and Radtke et al. (1990). Sadovy and Severin (1992) suggest that temperature was not the causative factor of Sr/Ca levels in previous studies; rather, it was an indicator of growth rate, which was positively correlated with temperature. For species such as Dover sole, which reside in upwelling systems where fastest growth occurs at cold temperatures, growth rate appears to over-ride temperature as a predictor of Sr/Ca ratios.

Postmetamorphic juveniles Microchemistry of otolith material deposited during Stage 5, prior to formation of the first post-settlement annulus, was identical to that formed following enclosure during Stages 3 and 4. The first and subsequent annuli were composed of translucent material deposited during the late fall and winter, with a high Sr/Ca ratio. During this period downwelling occurs, resulting in higher water temperature and lower nutrient concentrations than during the spring-summer upwelling season (Huyer et al., 1979; Landry et al., 1989). Kreuz et al. (1982) examined scales of adult Dover sole and noted that fastest growth occurred during the cold upwelling season, rather than during the warmer downwelling season, presumably because of higher productivity. The correlation of high Sr/Ca ratios and slow growth rate during formation of the first and subsequent post-settlement annuli is in agreement with observations of Sadovy and Severin (1992), as is the continued deposition of low Sr/Ca opaque material during subsequent seasons of rapid growth.

Summary of otolith landmarks

Several structural and chemical landmarks were correlated with metamorphic stages of Dover sole (Table 6). These landmarks were highly consistent among otoliths and, when coupled with information from daily growth increments, may be useful in determining timing of developmental and behavioral events associated with metamorphosis.

We conclude that the most consistent landmarks associated with the initiation event of metamorphosis (Markle et al., 1992), which includes movement of the left eye beyond the mid-dorsal ridge, are the transition from clear to opaque otolith material and formation of the first accessory primordium. Completion of eye migration apparently occurs between formation of the first and last AP, which encompasses a period of at

[2]J. Butler, S.W. Fisheries Science Center, NMFS, P.O. Box 271, La Jolla, CA 92038, pers. commun. 1992.

Table 6

Summary of larval and juvenile Dover sole, *Microstomus pacificus*, metamorphic stages (as described in text and Markle et al., 1992), structural landmarks on otoliths, chemical landmarks on otoliths, and habitat.

Stage	Structural landmarks	Chemical landmarks	Habitat
1	Translucent; all growth emanates from central primordium	Sr/Ca ratio high: 0.007–0.010 Spike and 0.004–0.006 "peak"	Pelagic; offshore from continental shelf
2	Opaque material; accessory primordia begin to form	Sr/Ca ratio declines from 0.004–0.006 to 0.002–0.003	Pelagic; moving inshore and towards bottom
End of 2 or beginning of 3	Opaque material; last accessory primordium forms; growth from accessory primordia encloses growth from central primordium	Sr/Ca ratio low, sometimes reaching minimum (≈0.002) near point of enclosure	First found on bottom; broad depth distribution on shelf and slope; also caught in midwater
3, 4, 5 Prior to first fall/winter on bottom	Opaque material; checks and left-right asymmetry become more prominent; rostrum forms	Sr/Ca ratio low (0.002–0.003)	Primarily benthic; narrow depth distribution on shelf; some Stage-3 and Stage-4 larvae also caught in midwater
5 Subsequent to first spring/summer on	Translucent material forming during fall/winter; opaque material forming during spring/summer	Sr/Ca ratio high (0.004–0.005) during fall/wiinter; low (0.002–0.003) during spring/summer	benthic

least 70 days in some individuals. The termination event of metamorphosis, as defined by Markle et al. (1992), is not associated with discrete structural or chemical signals.

We propose that the landmark most likely to correspond with the behavioral change of settlement from water column to benthos is enclosure of growth from the central primordium by growth from accessory primordia. In some individuals a minimal Sr/Ca value near 0.002 may also signal this event. This behavioral change is not instantaneous, because both Stage-3 and Stage-4 larvae were collected in midwater trawls. Markle et al. (1992) characterized settlement in Dover sole as a gradual process in which larvae make their way from an initial deep-water landing zone to a shallower nursery area. Enclosure of growth from the central primordium occurs near the beginning of Stage 3, when Dover sole larvae are first collected on the bottom in significant numbers. Thus, it is reasonable to expect that this is the point at which they first begin to spend a significant amount of time on the bottom.

Chronology of metamorphosis

Periodicity of increments emanating from the central primordium has not been validated, so assignment of dates to otolith landmarks formed during Stage 1 and Stage 2 can only be speculative. However, if increments are deposited approximately daily, then Stage-1 larvae reached a length of 20–30 mm in approximately 2–3 months, some Stage-1 larvae reached 46–50 mm in approximately 5–8 months, and most larvae reached the end of Stage 2 or beginning of Stage 3 about a year after increments first formed. These rates are approximately double those determined from seasonal collections of Dover sole larvae (Markle et al., 1992). Resolution of this discrepency must await validation of increment periodicity in premetamorphic larval otoliths.

Periodicity of growth increments was confirmed for Stage-3 and Stage-4 larvae and Stage-5 juveniles. In general, duration of these stages is in agreement with other studies. The duration of Stage 3 following enclosure averaged 28 days, although it extended at least 65 days for one individual. Because enclosure may oc-

cur during Stage 3, it is possible that total duration of Stage 3 is longer. Markle et al. (1992) suggested that, based on seasonal collections of Stage-3 and Stage-4 larvae, Stage 3 lasted approximately 45 days. Without a landmark for the Stage 3 to 4 transition, duration of Stage 4 could not be determined in this study. However, the combined period of Stages 3 and 4 could last up to 126 days, based on increments distal to enclosure of otoliths from Stage-4 larvae. Markle et al. (1992) observed one captive larva that took 43 days to progress through Stage 4, suggesting a combined duration through Stages 3 and 4 of about 90 days.

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Appendix

Origin of specimens examined for microstructure and microchemistry.

Stage	Number	Date(s)	Collection
1	1	January 1987	Midwater trawl; 300 km off Oregon; upper 200 m of water column
	2	March 1990	Midwater trawl; 75 and 532m bottom depth; upper 30 m of water column (Whipple, 1991[1])
	2	July 1991	Shrimp trawl; off Washington; 128 m depth
	1	October 1991	Shrimp trawl; off Oregon; ≈128 m depth
	Total: 6		
3	89	January 1989, March 1989, January 1990	Shrimp trawl with 6.4-mm liner; off Oregon; 55–377 m (Markle et al., 1992)
	14	March 1990	Midwater trawl; off northern California; bottom depths 73–1462 m; upper 110 m of water column (Whipple, 1991[1])
	Total: 103		
4	66	January 1989, March 1989, January 1990	Shrimp trawl with 6.4-mm liner; off Oregon; 40–170 m (Markle et al., 1992)
	16	March 1990	Midwater trawl; off northern California; bottom depths 33–91 m; upper 30 m of water column (Whipple, 1991[1])
	Total: 82		
5	220	January 1989, March 1989, January 1990	Shrimp trawl with 6.4-mm liner; off Oregon; 75–188 m (Markle et al., 1992)